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Small Molecule-Based Chemical Affinity System for the Purification of Proteins

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ABSTRACT

A new chemical affinity system is described for the purification of proteins. The Linx™ Affinity Purification System enables researchers to quickly and easily bind a protein ligand to a chromatographic matrix and use the resulting affinity resin to purify a second protein from crude mixtures. The entire process takes approximately 2 h.

INTRODUCTION

The use of immobilized affinity ligands on chromatographic supports for the purification of target molecules is perhaps the most common of all affinity techniques (1–3). To date, many affinity chromatography systems are comprised of activated supports requiring ligand-coupling procedures that are often complex and time consuming (2–5). We describe here the use of a new small molecule-based chemical affinity system, which allows the user to quickly and easily prepare an affinity resin for purifying proteins from crude mixtures.

The Linx™ Affinity Purification System (Prolinx, Bothell, WA, USA) is based on the specific interaction between two families of small molecules, the simplest representatives of which are phenylboronic acid (PBA) and salicylhydroxamic acid (SHA). These two molecules react to form a complex under a variety of conditions, the only by-product of which is an equivalent of water (Figure 1A). The complex is reversible and is in equilibrium with the reaction components; however, under the appropriate conditions of concentration, pH and temperature, the complex is highly favored. For affinity chromatography applications, a protein ligand is conjugated with phenyldiboronic acid (PDBA), a bivalent derivative of PBA (Figure 1B). The N-hydroxysuccinimide (NHS) ester on the PDBA reacts with nucleophilic ε-amine groups of the protein's solvent-accessible lysine residues. This results in the formation of a stable amide linkage between the PDBA and the protein. Similarly, an NHS ester derivative of SHA is used to conjugate the SHA to EAH Sepharose® 4B (Amersham Pharmacia Biotech, Piscataway, NJ, USA) utilizing the resin's primary amine groups and also resulting in the formation of a stable amide linkage. The PDBA-conjugated protein is then immobilized through the formation of the PDBA-SHA complex onto SHA Sepharose (Prolinx).

MATERIALS AND METHODS

Conjugation of Proteins with PDBA-NHS

Typically, protein solutions were prepared to final concentrations of approximately 1 mg/mL in 100 mM $NaHCO_3$, pH 8.5. One milligram of protein was conjugated in 1 mL 100 mM $NaHCO_3$, pH 8.5, at a molar input ratio of 15:1 to 100:1 PDBA-NHS (Prolinx) to protein. The PDBA-NHS solution (5 mg/mL in anhydrous dimethylformamide) was added to the protein solution, and the entire reaction was incubated for 30 min to 1 h at room temperature. The PDBA-labeled protein was then either added directly to SHA Sepharose or purified by gel filtration and analyzed for conjugation efficiency (the final number of PDBAs per protein).

Affinity Enrichment of a Polyclonal Antibody from Rabbit Serum

Immobilized RNase A was used to affinity purify anti-RNase antibodies from polyclonal rabbit serum. One milligram of PDBA-RNase A (molar input ratio of PDBA-NHS:protein, 100:1) was bound to 0.25 mL SHA Sepharose in a disposable 2-mL column. The affinity resin was charged by incubation with PDBA-RNase A by batch loading for 30

min with gentle rotation. The columns were then drained and washed two times with 5-mL aliquots of 0.1 M $NaHCO_3$, pH 8.0. All fractions were collected and used to determine binding efficiency (essentially 100% under these conditions).

Two milliliters of a polyclonal rabbit antiserum specific for RNase A were diluted into 10 mL 0.1 M $NaHCO_3$/0.5 M NaCl at pH 8.0 and then applied to the above column. The resin slurry was incubated for 30 min at room temperature to allow the antibody to bind to the affinity resin. The column was drained and washed first two times with 5-mL aliquots of 0.1 M $NaHCO_3$, 0.5 M NaCl at pH 8.0, followed by two times with 5-mL aliquots of 0.1 M $NaHCO_3$, pH 8.0, collecting and saving all fractions for later analysis. The antibody was eluted by capping the bottom of the column and adding 1 mL 100 mM $NaHCO_3$, pH 11.2. The elution buffer was mixed into the resin, incubated at room temperature for 1 min and allowed to drain into the first elution tube containing 50 µL neutralization buffer (1 M NaH_2PO_4, pH 5.5). Subsequent elutions were 1 mL each. As described above, these elutions were also captured in collection tubes containing neutralization buffer, but the mixing and incubating steps were omitted. A second set of elutions was done with each column by adding eight column volumes of 0.1 M glycine, pH 2.2. Fractions were examined for protein concentration (Bradford assay), composition (SDS-PAGE and protein stain) and specific binding activity with the target protein (ELISA).

Affinity Purification of an Epitope-Tagged Recombinant Protein from Bacterial Lysate

HB101 *E. coli* were transformed with pNUTCREBMyc-His (which expresses a 35-kDa single-chain antibody C terminally fused with a myc epitope tag and a poly His sequence from the lac promoter; Invitrogen, Carlsbad, CA, USA) and grown overnight on LB agar containing ampicillin. One colony was expanded in 1 L liquid LB/amp to mid-log phase (OD_{600} = 0.5) and induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 18 h. The culture was then centrifuged at 4000× *g* for 15 min, then the pellet was resuspended in 40 mL lysis buffer (50 mM KH_2PO_4, pH 7.8, 100 mM KCl, 10% glycerol, 0.5% Triton® X-100, 1 mM PMSF and 10 µM leupeptin). The resuspended cells were freeze-thawed once and sonicated 10 times for 30 s. The resulting crude lysate was then cleared by centrifugation at 9500× *g* for 10 min at 4°C.

To prepare an antibody affinity column, the His C terminal antibody (Invitrogen) was conjugated with PDBA-NHS at a molar input ratio (PDBA-NHS:antibody) of 100:1 in PBS, pH 7.4, for 30 min at room temperature. A 0.25-mL SHA Sepharose column was charged with 1 mg PDBA-antibody conjugate (unpurified) for 30 min at room temperature. The column was then drained and equilibrated with 6 mL 100 mM $NaHCO_3$, pH 7.0, then batch loaded with 0.25 mL cleared bacterial lysate (described above) diluted to 0.5 mL in PBS at a final pH of 7.0. The column was washed with 5 mL 100 mM $NaHCO_3$, pH 7.0, and the purified protein was eluted with 2.5 mL 100 mM $NaHCO_3$, 0.5 M imidazole, pH 7.0.

Determination of Buffer Effects on P(D)BA-SHA Binding

SHA Sepharose binding experiments were performed with PBA or PDBA [P(D)BA] conjugates of a commercially available polyclonal goat antibody, antimouse IgG Fc (Rockland Immunochemical, Gilbertsville, PA, USA). The conjugates

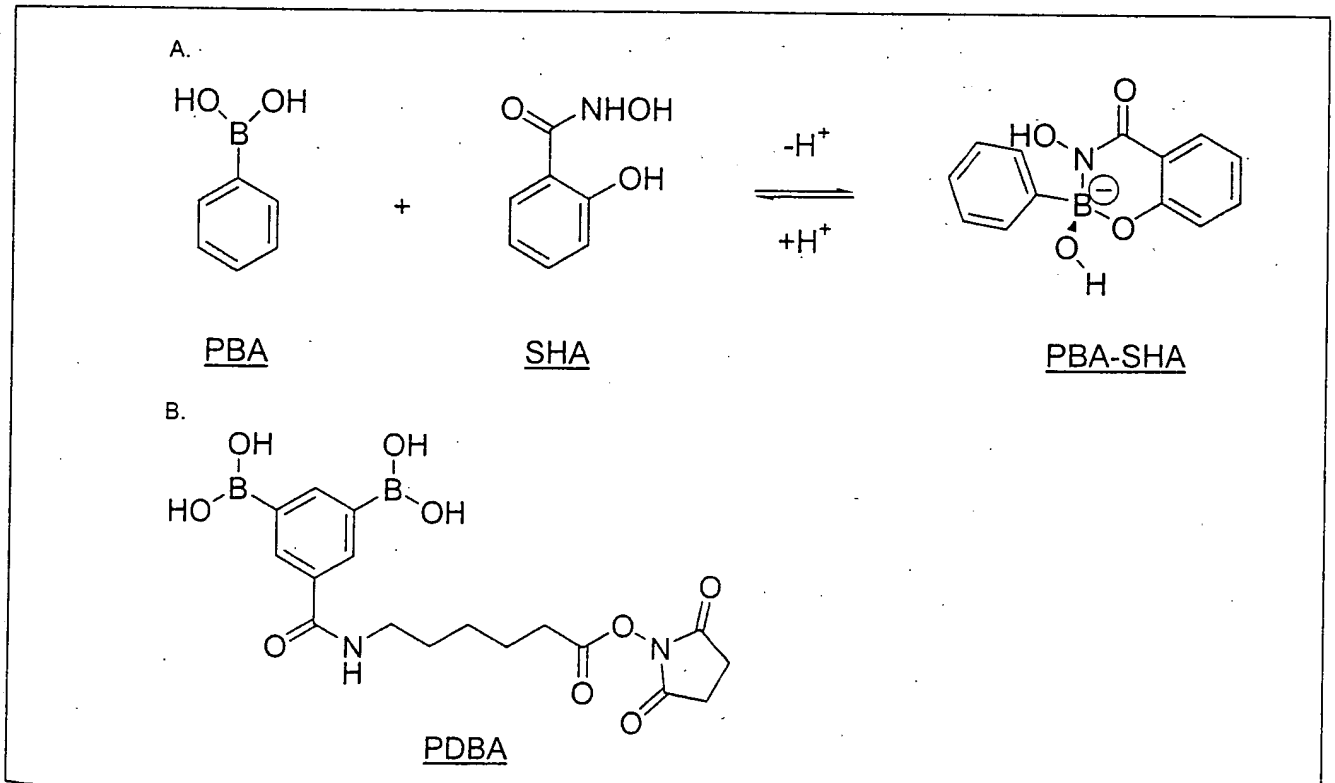

Figure 1. The PBA:SHA chemical affinity system. (A) The reaction of PBA with SHA. The SHA is covalently anchored to the surface of Sepharose, and a PBA derivative is conjugated to a protein of choice. (B) NHS ester of PDBA. The NHS ester derivative of PDBA is used to conjugate protein ligands.

were prepared by reacting the antibody at 10 mg/mL in 0.1 M $NaHCO_3$ with a tenfold molar excess of P(D)BA-NHS for 1 h at room temperature. After the reaction, unconjugated P(D)BA-NHS and its degradation products were separated from the antibody conjugate by desalting on a Sephadex® G-25 column (Amersham Pharmacia Biotech) in PBS buffer. The antibody-P(D)BA conjugate concentrations were estimated from their UV light absorbance at 280 nm, assuming 1 mg/mL to have an absorbance of 1.4. The A_{280} was not corrected for contribution from P(D)BA moieties.

The binding experiments were performed by column chromatography on a BioCAD® Sprint workstation (PerSeptive Biosystems, Framingham, MA, USA), except as noted, using gel beds 1 cm in diameter with a height of 2 cm (approximately 1.5-mL bed) at a flow rate of 2 mL/min (76 cm/h). Binding experiments not performed on the BioCAD were performed in open columns of the same dimensions at approximately the same flow rate. The columns were pre-equilibrated in the buffer condition to be evaluated. Two-milliliter samples containing 0.75 mg P(D)BA conjugate in test solution were applied to the column in a volume of 2 mL, followed by washing the column in test buffer and elution with acetic acid.

The amount of conjugate binding to the column was calculated from the absorbance trace as the ratio of the integrated area of the material eluted from the column by 0.2 M acetic acid to the total area of all material flowing through or eluted from the column. Large background absorbance by some of the buffer components prevented accurate measurements of the relative fraction of conjugate bound by the column. In these cases, the extent of binding was approximated, but it is still believed to be an accurate indication of performance.

Effect of Common Sterilization Procedures, Antimicrobial Agents and RNase Treatment on SHA Sepharose Capacity

For chemical sterilization, 0.90 mL SHA Sepharose was packed into an Econo Column® (1 × 10 cm; Bio-Rad Laboratories, Hercules, CA, USA) and treated with 20 mL 70% ethanol in water. The flow was stopped, and the column was sealed with approximately 2 mL ethanol/water solution remaining on the head of the column. The column was stored at room temperature overnight (approximately 20 h). The column was then re-equilibrated with 20 mL PBS (140 mM NaCl, 8 mM $NaHPO_4$, 2 mM NaH_2PO_4, pH 7.3), and the capacity for N-(3-dihydroxyborylphenyl)-N′-(6-hydroxyhexyl) butane-1, 4-diamide, PBA-XX-OH, (Prolinx) was determined as described below. Alternatively, SHA Sepharose in 20% (v/v) ethanol in water was autoclaved (20-min cycle: T = 127°C; 17 psi) in an amber glass bottle. The column was re-equilibrated with 20 mL PBS, and the capacity of the SHA Sepharose for PBA-XX-OH was determined.

The effect of a typical antimicrobial agent, chlorhexidine digluconate (Sigma, St. Louis, MO, USA), was examined. Two SHA Sepharose columns were prepared by packing 1.41 and 1.10 mL media into Econo columns. The columns were equilibrated with 40 mL aqueous solution comprised of 2% chlorhexidine digluconate and 20% denatured ethanol. The flow was then stopped, and the columns were stoppered with approximately 2 mL chlorhexidine digluconate solution remaining on the head of the columns. The columns remained at room temperature for 6 h, following which they were re-equilibrated with 100 mL PBS, and the capacity for PBA-XX-OH was measured.

To remove any RNase contamination, SHA Sepharose was treated with diethylpyrocarbonate (DEPC; Sigma). Approximately 4 mL SHA Sepharose were rinsed with 10 mL water. The wet slurry was transferred to an amber glass bottle, and 20 mL 0.1% (vol/vol) DEPC in water was added. The bottle was then capped and stored at 37°C overnight. After 20 h, the SHA Sepharose was divided into two lots. One lot was equilibrated with 100 mL PBS and assayed for capacity, as described below. The second lot was subjected to an autoclave cycle (20 min, 127°C, 17 psi) before determining its capacity.

The capacity of SHA Sepharose was determined by preparing a 1 × 1.25 cm (± 0.35 cm) column. The column was connected to an Econo UV Monitor with a 280-nm filter (Bio-Rad Laboratories) and rinsed with at least 20 mL PBS. A 0.1-M stock solution of PBA-XX-OH was prepared in dimethylformamide. A working solution of PBA-XX-OH was prepared by diluting 150 µL stock solution to 15 mL with PBS. The working solution of PBA-XX-OH was applied to the column until the recorder detected a sharp rise in the baseline (a tared beaker was used for eluent collection). The total volume of PBA-XX-OH applied to the column was noted. The PBS buffer solution was then passed through the column until the peak eluted in its entirety (returned to baseline). The weight of the eluent solution was noted. The UV absorbance of both the working solution of PBA-XX-OH and the eluent was determined at 260 nm ($\varepsilon = 4500\ M^{-1}cm^{-1}$). The capacity of the SHA Sepharose was determined by difference, using the weight (volume) of eluent, the volume of working solution passed through the column, the volume of the resin in the column and the concentration of PBA-XX-OH in both working solution and eluent. It is estimated that a single capacity measurement determined by this method has a standard error of ±0.5 µmol/mL.

Side-by-Side Comparison of the Linx Affinity Purification System with Commercially Available NHS Esters of a Derivatized Cross-Linked Agarose Gels

The ligand-binding and target molecule recovery efficiencies of the Linx Affinity Purification System were compared with two versions of commercially available NHS ester-linked agarose supports. The two competitive products ("Competitive Product A" and "Competitive Product B") differed from each other in the chemical identity of the linker arm connecting the NHS ester to the agarose.

The immobilized protein was calf intestine alkaline phosphatase (Sigma), with a pI in the range of 4.4–5.8. The alkaline phosphatase was dialyzed into 100 mM sodium bicarbonate (pH 8.3) before use in all of the experiments. The biological molecule targeted for cleanup was polyclonal alkaline phosphatase (Calf Intestine, Rabbit; Rockland, Gilbertsville, PA, USA). All experiments were performed with 0.25 mL solid support.

For the Linx Affinity Purification System, PDBA-X-alkaline phosphatase was prepared from a 50:1 input ratio of PDBA-X-NHS to alkaline phosphatase. The experiment was performed in duplicate with 3 and 4 mg protein. The conjugation reaction proceeded on ice for 1 h. No cleanup of the conjugate was performed before introduction to the Econo column (15 × 0.5 cm, filled with 0.25 mL SHA Sepharose and equilibrated with 100 mM sodium bicarbonate). The application of conjugate to the

column was followed using a Model EM-1 Econo UV Monitor with a 280-nm filter (Bio-Rad Laboratories). The column was rinsed with a total of 10 mL 100 mM sodium bicarbonate until the baseline returned to zero. The eluent was dialyzed versus 100 mM sodium bicarbonate to remove unreacted conjugation reagent and by-products. The protein conjugate remaining in the eluent was quantitated using UV spectroscopy.

Immobilization of alkaline phosphatase on Competitive Products A and B were each performed in duplicate. Competitive Product (0.25 mL solids basis) was placed in a 1.5-mL Eppendorf® tube. The solids were washed three times with cold deionized water, each time using microcentrifugation to separate solids and liquid. The liquid was removed between washings. Approximately 0.5 mL 100 mM sodium bicarbonate containing alkaline phosphatase was added to each tube. For Competitive Product A, Trial 1, 3 mg alkaline phosphatase was added, and the tube was spun end over end at room temperature for 1 h. For Competitive Product A, Trial 2, 4 mg alkaline phosphatase was added, and the tube was spun end over end at room temperature for 2 h. For Competitive Product B, Trial 1, 3 mg alkaline phosphatase was added, and the tube was spun end over end at room temperature for 1 h. For Competitive Product B, Trial 2, 4 mg alkaline phosphatase was added, and the tube was spun end over end at 4°C for 4 h. Following reaction of protein with ligand, 0.025 mL 1 M glycine ethyl ester (Sigma), pH 8.0, was added to each tube to block any unreacted NHS esters. The tubes were spun end over end for an additional hour.

Following the blocking reaction of Competitive Products A and B, each aliquot of packing material was quantitatively transferred to an Econo column, 15 × 0.5 cm. The eluant was collected, and the column was washed with sodium bicarbonate to collect a total of 10 mL. The eluant was dialyzed to remove reaction by-products, and then the protein in the eluant was quantitated by UV spectroscopy. The amount of protein remaining on the column was calculated by difference.

For all experiments (Linx Affinity Purification System and Competitive Products A and B), the protocol for the application and recovery of alkaline phosphatase was the same. Each column containing support and attached ligand was connected to the A280 Monitor. A total of 20 mg antibody was introduced to the column in a volume of 5 mL. The column was eluted (back to baseline) with 100 mM sodium bicarbonate. A total of 15 mL eluant was collected. The concentration of the antibody in the eluant was determined by UV spectroscopy, and the amount retained by the column was calculated by difference. Recovery of the antibody from each affinity column was by elution with 9 mL high-pH (phosphate, pH 11.0) buffer. The buffer was collected in a 15-mL tube containing 1 mL 1 M phosphate buffer, pH 5.75. The concentration of antibody in the high-pH rinses was determined by UV spectroscopy. The high-pH rinsings of each column were examined by 12.5% SDS-PAGE (silver stained) to verify that the eluant contained anti-alkaline phosphatase and not alkaline phosphatase.

RESULTS AND DISCUSSION

Affinity Enrichment of a Polyclonal Antibody from Rabbit Serum

The Linx Affinity Purification System was designed to offer the researcher a fast and efficient method of generating

Table 1. Purification of Rabbit Anti-RNase

Sample	Total Protein	Total Units	U/mg	Fold Enrichment	Recovery
Crude Serum	56 mg	33092	591	—	—
Affinity Purified (pH 11.2 elution)	0.19 mg	12460	64896	110	38%

Anti-RNAse A-specific activity enrichment (determined by immunoreactivity) and total recovery after affinity purification. The activity units reported here represent the dilution of the sample required to give an OD_{405} of 0.5 in an RNase ELISA.

solid-phase matrices for use in affinity chromatography. As an example, we show here that this system can be used for the affinity purification of a specific antibody from a crude polyclonal mixture. First, RNase A was modified with PDBA and bound to SHA Sepharose, creating an RNase affinity column in less than 1 h. Over the column was passed rabbit antiserum raised against RNase as described in the Materials and Methods section. Then high- and low-salt washes were performed at pH 8.0. After the wash steps, the proteins were eluted with pH 11.0 (Figure 2, lane 3), then eluted with pH 2.2 (Figure 2, lane 4). Column fractions were neutralized then run on 4%–20% polyacrylamide gels and silver stained. As demonstrated here, the fraction eluted from the resin by either pH 11.0 or by pH 2.0 is highly enriched for immunoglobulin heavy (H) and light (L) chain compared to the input rabbit serum (Figure 2, lane 2). The fractions were also tested for activity by an ELISA titration assay. As reported in Table 1, antibody activity specific for RNase was enriched by 110-fold over that in the crude serum with 38% recovery of total activity. In these experiments (and in data not shown), we noticed a small amount of protein that bound to the SHA resin nonspecifically and eluted under high pH conditions (pH 11.0). The contamination was minimal (detectable on an overloaded, silver-stained PAGE gel) and can be reduced or eliminated by using more stringent wash conditions (pH 9.0) or by the use of a competitive eluant (see the example below using imidazole).

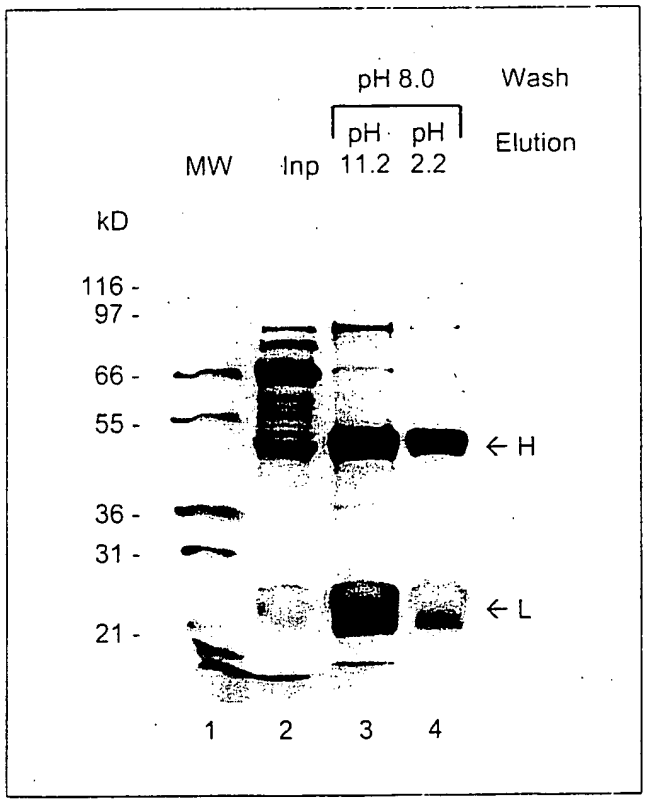

Figure 2. Purification of rabbit anti-RNase A. Silver-stained PAGE of crude input (lane 2) and eluted fractions (lanes 3 and 4) from crude rabbit anti-RNase A serum purified on a RNase affinity column (H = Ig heavy chain, L = Ig light chain).

Affinity Purification of an Epitope-Tagged Recombinant Protein from Bacterial Lysate

In a second example, an antibody directed toward C-terminal His tags was used to affinity purify a single-chain antibody containing a C-terminal poly His tag (CREB sFv). The C-terminal His antibody has limited and pH-dependent affin-

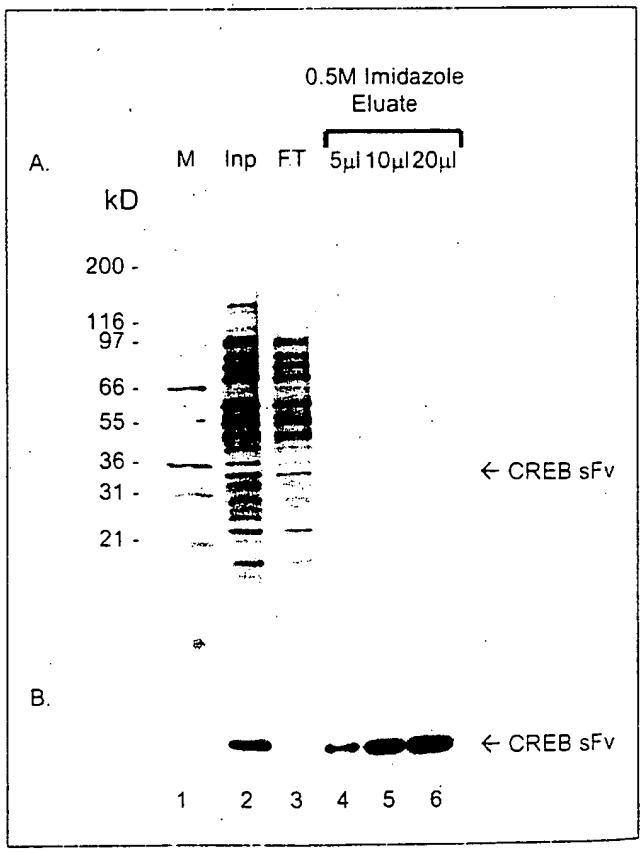

Figure 3. Purification of a C-terminal His-tagged single-chain antibody. (A) Silver-stained PAGE of crude input (Inp. 0.5 µL), flow-through (FT. 0.5 µL) and the purified protein fractions eluted with 0.5 M imidazole. (B) Anti-His immunoblot detected with anti-C-terminal His HRP conjugate (same fractions as described in panel A).

Table 2. Analysis of Protein Conjugation Number and Resistance to Elution at High pH

Protein	PDBA-NHS:Protein (molar input ratio)	Conjugation Number (final)	Elution of RNase at pH 11.2 (method of detection)	
RNase A	5:1	0.66	yes	(PAGE)
RNase A	15:1	1.56	trace	(PAGE)
			yes	(activity)
RNase A	45:1	4.00	not detected	(PAGE)
			trace	(activity)
RNase A	100:1	N.D.[a]	not detected	(PAGE)
			not detected	(activity)
BSA	5:1	2.25	trace	(PAGE)
BSA	15:1	4.00	not detected	(PAGE)
BSA	45:1	8.60	not detected	(PAGE)

[a]Conjugation number not determined because of potential interference of PDBA with Bradford and BCA reagents.

ity for natively folded His-tagged proteins but binds well to denatured proteins in Western blots. In the case of the anti-CREB sFv, the pH of the input and wash was kept at 7.0. Elution of the fraction containing the His-tagged sFv was achieved with the histidine competitor, imidazole, at 0.5 M. The pooled fraction of the eluted peak (1 mL) contained 40 µg CREB sFv at greater than 90% purity and was approximately 150-fold purified from the crude lysate (6 mg) (Figure 3, panel A). Western blot analysis using the anti-His C-terminal horseradish peroxidase (HRP) antibody conjugate (Invitrogen) (Figure 3, panel B, lanes 2 and 3) shows that under these conditions, the anti-His affinity column efficiently depletes the extract of CREB sFv. The entire procedure, from conjugation of the C-terminal His antibody to collection of the purified fraction of sFv was completed in a total of 2 h.

The Effect of Buffer Conditions on PBA-SHA Binding

In most affinity chromatography applications, washing the bound complexes with various buffer conditions is of prime importance to determine real from nonspecific interactions. As leeching of bound "target" is a common problem with affinity matricies. We tested the Linx system for binding under a variety of buffer conditions. The binding characteristics of PBA and PDBA are comparable. The contributions of multivalency (whether it arises from the use of PDBA or by increasing the conjugation number) has a positive effect on avidity and therefore increases the binding efficiency of the conjugated protein. Thus, some initial experimentation to optimize the level of PDBA conjugation per ligand permits preparation of a matrix with optimum immobilization characteristics without any loss in binding affinity to the biomolecule being purified.

The data resulting from the examination of buffer conditions on P(D)BA-SHA binding illustrated that the P(D)BA-SHA bond is stable across a broad range of pH conditions and in relatively complex solutions containing moderate quantities of detergents, denaturants, salts and chelating agents. In summary, the P(D)BA-SHA bond is stable to pH ranges of 5.0–9.0. Amine-containing buffers such as Tris or glycyl-

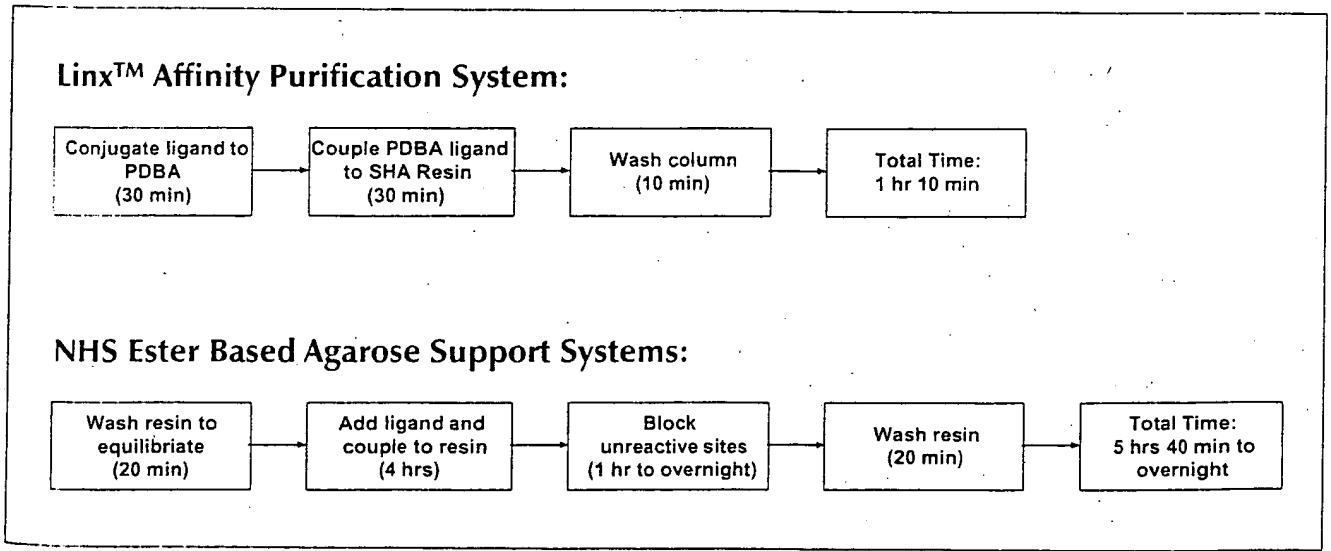

Figure 4. Efficiency of PDBA-SHA affinity column preparation. The Prolinx affinity chromatography system requires significantly less time from start to finish than an NHS ester-based solid support system such as Competitive Products A and B described in this paper.

Table 3. Effect of Common Sterilization Procedures, Antimicrobial Agents and DEPC Treatment on SHA Sepharose Capacity

Treatment	Capacity Before Treatment (µmol/cm³)	Capacity After Treatment (µmol/cm³)	Difference (µmol/cm³)	Percent Change
Ethanol	4.1	3.7	0.4	-9.7%
Autoclave	4.1	3.6	0.5	-12.2%
Chlorhexidine (1)	3.7	3.0	0.7	-18.9%
Chlorhexidine (2)	3.7	3.2	0.5	-13.5%
DEPC	4.3	2.9	1.4	-32.6%
DEPC + Autoclave	4.3	3.2	1.1	-25.6%

Standard error for single point measurement estimated at ±0.5 µmol/cm³.

glycine can inhibit the formation of the complex to some extent and are not recommended during the immobilization step. However, once the ligand has been immobilized, the complex exhibits increased stability, and a greater variety of buffers may be used to wash and elute the affinity column including those containing amines. The complex also exhibits good stability at high ionic strength. Concentrations of NaCl up to 1.5 M, $MgCl_2$ concentrations up to 100 mM and concentrations of EDTA over 10 mM do not interfere with P(D)BA:SHA stability. High concentrations of denaturants such as 4–8 M urea and guanidine (>2 M) also do not appear to affect P(D)BA-SHA binding to any appreciable extent. Examples of conditions that may negatively effect P(D)BA-SHA bond formation include: sodium acetate buffer, pH 4.0 (but not at pH 5.0), high concentrations (3.0 M) of guanidine, high concentrations (3%) of Triton X-100 and high concentrations (2%) of SDS. Increasing the valency by using PDBA and increasing the number of PDBA molecules per target protein stabilizes the protein binding to the SHA Sepharose, thereby making its binding more resistant to harsh conditions (see below).

The Effect of PDBA Conjugation Number and Antigen Resistance to High-pH Eluants

In many cases, the use of harsh elution conditions is necessary to break an antibody-antigen interaction to recover the purified antibody of interest. For example, extremes of pH at 2.5 and 11.5 can be used to effectively elute most antibodies in an active state. Since the PDBA-SHA bond is, to some extent, pH sensitive, these harsh conditions may disrupt PDBA-SHA binding and cause the immobilized PDBA-antigen to co-elute with the antibody. The problem can be minimized by increasing the molar input ratio of PDBA-NHS to protein (antigen), resulting in an increase in the final PDBA conjugation number (the average number of PDBA molecules per protein molecule). Increasing the PDBA conjugation number serves to increase the apparent affinity of the PDBA-modified protein to the SHA column by increasing avidity. Table 2 illustrates the effect that an increase in the PDBA conjugation number has on the stability of the PDBA-SHA interaction used to immobilize the protein to the solid support.

As the data in Table 2 indicate, increasing the input ratio of PDBA-NHS to protein allows the antigen to bind tighter to the SHA resin, as indicated by increased stability to high-pH elution conditions. Examination and analysis of the conjugation numbers, PAGE gels and RNase activity assays show a good correlation between an increase in the conjugation number and retention of the antigen on the SHA Sepharose. High levels of substitution may decrease enzymatic activity of the RNase, thereby making protein leeching from the column undetectable by the assay used here. The protein was still able to specifically bind antibody, and leeched RNase (if any) was undetectable on an overloaded silver-stained PAGE gel. If less harsh elution conditions can be used (e.g., a competitive ligand), higher levels of PDBA conjugation of the target protein may not be necessary or desirable.

Determination of the Capacity of SHA Sepharose for PDBA-Conjugated BSA

BSA was conjugated at a molar input ratio of 15:1 (PDBA-NHS:BSA) and purified by gel filtration (G-25, 1 × 10 cm). Fifteen milligrams of the conjugate in 100 mM $NaHCO_3$, pH 8.0, was loaded onto a 0.5-mL SHA Sepharose column (resin capacity measured at 6.9 µmol PBA-XX-OH bound/mL resin) while collecting 1-mL flow-through fractions. Unbound protein was not detected until a total of 14 mg had been loaded. The remaining 1 mg was accounted for in the final flow-through fraction and wash. Therefore, the capacity of SHA Sepharose under the conditions described here is 28 mg (approximately 400 nmol) PDBA-BSA/mL SHA Sepharose resin.

Effect of Common Sterilization Procedures, Antimicrobial Agents and RNase Treatment on SHA Sepharose Capacity

Some applications of the Linx Affinity Purification System may require the SHA Sepharose to undergo sterilization or DEPC treatment (for removal of RNase activity) before use. Table 3 illustrates the effect of two sterilization procedures, a common antimicrobial reagent and DEPC treatment on SHA Sepharose as measured by its binding capacity for PBA-X-X-OH. The capacity remains essentially unchanged (within experimental error) after ethanol sterilization, autoclaving and treatment with the antimicrobial reagent chlorhexidine (hibitane) digluconate. The capacity of the resin for PBA-X-X-OH is reduced by approximately 30% following treatment with DEPC. These data taken together show that the SHA Sepharose is resistant to common sterilization procedures and only loses partial activity when treated for RNase contamination.

Table 4. Side-by-Side Comparison of Linx Affinity Purification System with NHS-Based Agarose Affinity Systems

Column Type	mg AP Applied	% AP Retained	mg α-AP Applied	% α-AP Retained	mg α-AP Eluted pH 11.0	Total Percent α-AP Recovered
Linx Affinity System	3	74	23	27	3.6	56
Linx Affinity System	4	73	22	24	3.2	62
Competitive Product A (1 h, room temperature)	3	0	23	14	1.1	32
Competitive Product A (2 h, room temperature)	4	38	22	18	1.1	28
Competitive Product B (1 h, room temperature)	3	33	20	2	trace	trace
Competitive Product B (4 h, 4°C)	4	25	19	8	0.76	48

Results of Side-by-Side Comparison of the Linx Affinity Purification System with Commercially Available NHS Esters of Derivatized Cross-Linked Agarose Gels

A side-by-side comparison of the Linx Affinity Purification System with commercially available NHS esters of derivatized cross-linked agarose gels revealed that, under the conditions studied, the Linx Affinity Purification System was clearly superior in both time requirements and performance (Figure 4 and Table 4). The Linx Affinity Purification System can achieve a purification in a minimum of 1 h and 10 min, while the Competitive Products require 5 h and 40 min to overnight for the same cleanup.

Competitive Product A contained a neutral 10 atom spacer arm and Competitive Product B contained a 15 atom spacer arm that incorporated a positive charge. While both supports were advertised to achieve coupling at pH 3.0–10.0, it was noted in product literature that Competitive Product A was most effective in coupling proteins near or below their pI, and Competitve Product B was most effective in coupling proteins near or above their pI. Because the pI of alkaline phosphatase is near 5, and sodium bicarbonate was used as the buffer (pH 8.0), it was thought that Competitive Product B would be superior to Competitive Product A. However, this was clearly not the case, even in the experiment in which the protein was reacted with Competitive Product B for 4 h at 4°C (Figure 4). The loading efficiency of the Linx Affinity Purification System was clearly two times better than the Competitive Products. The subsequent recovery of antibody for the SHA Sepharose was over three times higher than for either of the competitive systems.

Affinity support media with attached NHS esters are unstable because of the reactivity of the NHS and require strictly anhydrous storage conditions. During the reaction of protein (affinity ligand) with an NHS ester-based affinity support, water competes with the protein for the NHS ester. SHA Sepharose, on the other hand, is stable when stored in aqueous media. Attachment of the P(D)BA affinity ligand to the SHA Sepharose column can therefore be nearly 100% complete.

In conclusion, the Linx Affinity Purification System is a fast, efficient method for the generation of an affinity matrix. This system allows the researcher to modify a target protein using common *N*-hydroxysuccinimidyl activation chemistry and subsequently immobilize it on an SHA-charged solid support in approximately 1 h. Once the affinity support is made, it can be used for a variety of affinity chromatography applications such as purification of proteins from crude mixtures. Furthermore, we have shown here that the PBA-SHA bond is stable to a variety of conditions such as high concentrations of salt, detergent and denaturant. Finally, the SHA-resin has a high capacity for PDBA-labeled proteins and is resistant to common sterilization and anti-RNase treatments.

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